Novel Molecule and Diagnostic Method

The invention relates to a dimer of HLA-B27 heavy chains or fragments thereof retaining the extracellular domain portions and use of such a dimer in a method of diagnosing or treating a spondyloarthropathy.

The association of HLA-B27 with spondyloarthropathy has been known for 25 years. 97% of patients with spondyloarthritis carry HLA-B27, compared to only 6% of caucasian controls. Although HLA-B27 is therefore implicated as a factor which influences susceptibility to spondyloarthropathy, the exact role played by HLA-B27 in disease aetiology remains unknown.

Currently, spondyloarthropathy is diagnosed on the basis of criteria which can only be detected during the later stages of disease. For instance, the most widely used criteria for diagnosing ankylosing spondylitis rely on radiographic evidence of sacroilitis. This is not always easy to interpret in the early stages of the disease and diagnosis can therefore be delayed for several years. Ankylosing spondylitis (AS) is a particularly difficult spondyloarthropathy to diagnose because the blood tests widely used for rheumatoid arthritis (erythrocyte sedimentation rate (ESR) and C reactive protein (CRP)) do not work in 50% of AS patients. The presence of HLA-B27 in the patient is not diagnostic in itself as it is also present in many healthy controls.

NSAIDs (non-steroidal anti-flammatory drugs), local steroids and sulphasalazine which are currently used to treat spondyloarthropathy are not disease-specific and have only minor long-term benefits.

The inventors have found an alternative form of HLA-B27 which is a homodimer of native HLA-B27 heavy chains held together by a disulphide bond between Cys 67 of each chain. This dimer of HLA-B27 heavy chains has been called 'HC-B27'. The inventors have further developed a diagnostic assay based on this finding. The diagnostic assay measures the level of a receptor which specifically binds to HC-B27. The levels of receptor bearing cells are found to be increased amongst ankylosing spondylitis patients. The level of receptor bearing cells is also found to increase during spondyloarthropathy attacks.

Thus the invention provides a substantially isolated dimer comprising first

and second cross-linked polypeptides, wherein said polypeptides comprise the extracellular domain portions of the HLA-B27 heavy chain and are capable of binding a HLA-B27 restricted epitope, or a substantially isolated functional dimeric or multimeric analogue thereof which is capable of binding said HLA-B27 restricted epitope and/or competes for binding to a specific receptor for said dimer.

The invention further provides a complex comprising biotinylated dimers bound to fluorescently labelled streptavidin in a molar ratio of 4:1.

The invention also provides a method of determining the onset of or predisposition to a spondyloarthropathy comprising measuring the level of or detecting the presence of a receptor in the human or animal body which binds to a dimer of the invention. The invention further provides a monoclonal antibody which binds a dimer of the invention, but does not bind to native HLA-B27.

The invention also provides a method of determining in a sample the presence of a substance which inhibits the binding of a dimer or complex of the invention with an antibody of the invention comprising:

(i) contacting said sample with said dimer or complex in the presence of said antibody; and

(ii) determining whether binding of said antibody to said dimer or complex is inhibited.

The invention additionally provides a dimer, complex, monoclonal antibody or a substance determined by the method above for use in a method of treating a spondyloarthropathy or for use as a prophylactic to prevent the onset of a spondyloarthropathy.

The invention also provides a method of determining the onset of or predisposition to a spondyloarthropathy which comprises measuring the level of or detecting the presence of HC-B27 in the human or animal body or in a sample from the human or animal body.

The term 'HLA' stands for human leukocyte antigen. In this specification HLA-B27 refers not only to the human MHC Class I molecule designated HLA-B27 but also to the equivalent MHC Class I molecule in an animal. The invention is applicable to all subtypes of HLA-B27.

The term "substantially isolated dimer" includes a dimer which is substantially free of other cellular components. This term also includes a substantially pure dimer which is present at at least 50%, 60%, 70%, 80%, 90%, 95% or higher purity by dry weight.

The HC-B27 specific receptor includes both cell-borne and soluble forms of the receptor. Thus the term 'the level of the receptor' may include the level of receptor bearing cells.

An HLA-B27 restricted epitope is one which binds in the peptide binding groove of the native HLA-B27 molecule. The polypeptide of the dimer is capable of binding a HLA-B27 restricted epitope. Therefore the polypeptide may have a conformation which is substantially similar to the conformation of a correctly folded HLA molecule. Such a conformation may be recognised by an antibody which recognises correctly folded HLA-B27 heavy chains, such as W6/32 antibody (obtainable from the ATCC). One or both of the polypeptides in the dimer may be capable of binding such an antibody. Thus the dimer may also be capable of binding a conformational antibody which recognises correctly folded HLA-B27 heavy chains, such as W6/32 antibody (W6/32 antibody recognises a conformational epitope in the $\alpha 2$ helix of the peptide binding groove). In one embodiment the dimer does not bind the conformational antibody ME1.

An antibody which recognises correctly folded HLA-B27 heavy chain can be produced using standard techniques, such as immunising an animal with correctly-folded HLA-B27, extracting B cells from the animal, fusing the B cells to immortalised cells, selecting fused cells based on their ability to produce antibody which binds correctly folded HLA-B27 and which does not bind incorrectly folded HLA-B27 and obtaining the antibody from the selected cells.

One or both of the polypeptides in the dimer may carry an HLA-B27 restricted epitope. Generally the dimer does not bind β_2-microglobulin (β_2m). The dimer may be one which binds or does not bind a lipid membrane.

When present in a human or animal the dimer may lead to the induction of a response. Such a response may comprise the production of or increase in the level of a receptor which binds specifically to the dimer, and therefore may be detected in the

diagnostic assay of the invention. The response may lead to the development of a spondyloarthropathy in a human or animal. In order to induce such a response the dimer may be administered to the human or animal. The dimer may also be administered to an experimental animal to induce a spondyloarthropathy. The human or animal may be HLA-B27 positive. Alternatively a transgenic animal could be produced which was engineered to express the dimer.

The dimer which is a functional analogue may bind one or two HLA-B27 restricted epitopes. This dimer may mimic the surface characteristics of the dimer or HC-B27. Therefore the analogue may bind to a receptor which is specific for the dimer, and thus competes with the dimer for binding the specific receptor. Thus the presence of the analogue may lead to a decrease in the measured level of binding between the dimer and a specific receptor.

The specific receptor may be an antibody of the invention as discussed below. The specific receptor may be the receptor which is present at increased levels in spondyloarthropathy patients.

The HLA-B27 restricted epitope is one which binds the native HLA-B27 molecule. Many examples of such epitopes are known in the art, such as influenza nucleoprotein (NP) epitope SRYWAIRTR (residues 383-391) or HIV gag epitope KRWIIMGLNK (residues 263-272). In one embodiment the dimer is able to bind this HIV epitope, but does not bind this influenza epitope.

The sequence of the polypeptide of the dimer may comprise the sequence of the extracellular domain portion of HLA-B27 heavy chain, more particularly residues 1 to 275 of the heavy chain, or a variant which has homology with the heavy chain. In the case where the polypeptide chain has homology with HLA-B27 heavy chain residues with positions functionally homologous to 1 to 275 are preferred.

The polypeptide is generally at least 70% homologous to the extracellular domain portion of the HLA-B27 heavy chain preferably at least 95%, 97% or 99% homologous thereto over the region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. Methods of measuring polypeptide homology are well known in the art, and it will be understood that the homology is calculated on the basis of amino acid identity. Particular algorithms

which may be used to calculate homology are discussed below.

The sequence of the extracellular domain portion can thus be modified for use in the dimer. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Insertions and deletions of amino acids can also be made. At least 1, 2, 3, 10, 10, 30 or more substitutions and/or at least 1, 2, 3, 10, 20, 30 or more insertions and/or at least 1, 2, 3, 10, 20, 30 or more deletions may be made.

The polypeptide may be a fragment of the extracellular domain or variant thereof (e.g. the homologous peptide discussed above). Preferably the extracellular domain portion includes at least the first two N-terminal domains of the HLA-B27 heavy chain. Therefore the extracellular domain portion may consist of residues 1 to 240, preferably 1 to 180, of the heavy chain. In the case where the polypeptide chain has homology with HLA-B27 heavy chain the chain may comprise or consist of residues functionally homologous to 1 to 240, preferably 1 to 180.

The polypeptide may be a modified version of the extracellular domain portion or modified versions of the fragments of the extracellular domain portion which have been mentioned previously. Such modifications may be natural post-translational modifications or artificial modifications.

The polypeptide chains in the dimer are preferably linked by the residue at position 67 of the heavy chain. In the case where a polypeptide chain has homology with HLA-B27 the preferred position is functionally homologous to position 67.

The polypeptide chains in the dimer are preferably cross-linked by a disulphide bond between Cys residues in the polypeptide.

The first and second polypeptides in the dimer may be the same or different.

The dimer may be linked to a detectable label, such as a radioactive or fluorescent label.

Dimers of the invention may be combined into larger multimeric complexes. If, for example, the binding of a substance to the dimer is to be measured then joining two or more dimers together would generally increase the sensitivity of the measurement. Thus, the dimer may comprise a moiety which allows two or more dimers to be linked to form a larger multimeric complex, such as a tetramer which consists of four dimers. Such a moiety may be biotin, as several biotin moieties are able to bind a single streptavidin molecule. A polypeptide of the dimer may comprise a BirA specific biotinylation sequence, such as GSLHHILDAQKMVWNHR. Such a sequence is recognised and biotinylated by BirA enzyme. In the presence of streptavidin the dimers may then form a tetramer through a biotin-streptavidin-biotin linkage. The dimers may be linked together through disulphide bonds, such as disulphide bonds through cysteines present in the cytoplasmic tail of the heavy chain.

In one embodiment the multimeric form comprises two dimers and is the same as the multimeric form which is produced when the heavy chains are expressed in T2 cells.

Generally the dimers in the complex will be the same, although they can be different.

In the case where a complex comprises a dimer comprising polypeptides which are extracellular domain portions of HLA-B27 heavy chain the polypeptide of the dimer will still be capable of binding a HLA-B27 restricted epitope. In the case where a complex comprises a dimer which is a functional analogue the analogue would still be capable of binding a HLA-B27 restricted epitope and/or competing for

binding to a specific receptor for the dimer.

The complexes may be linked to a detectable label, such as a radioactive or fluorescent label.

In the case where the dimers in a complex are linked together through a streptavidin molecule, the streptavidin molecule may be labelled. Such a label may be a fluorescent label.

The invention also provides a method of making a dimer of the invention which comprises providing a first polypeptide and a second polypeptide in conditions where they cross-link. The polypeptides may be expressed in a cell of the invention. Such a cell may be a human or animal cell. The cell may be one which naturally expresses the polypeptide. Alternatively the cell may be transfected with a nucleic acid which encodes one or both of the polypeptides. Thus the polypeptides may be expressed in a recombinant system. The cell may be an ex-vivo cell. Preferably the cell does not express β_2-microglobulin.

The dimer or complex may be used to detect the presence of a substance which binds to the dimer or complex. Thus the invention provides a method of measuring the level or detecting the presence of a substance which binds the dimer or complex comprising:

(i) contacting the sample with the dimer or complex; and

(ii) measuring the binding of the substance with the dimer or complex.

The binding of the substance to the dimer or complex may be measured in a competition assay. Such an assay measures the inhibition in the binding of the dimer or complex to a known amount of an entity which binds the dimer or complex in the presence of the sample. The entity may be an antibody of the invention.

A receptor produced in the human or animal body which is specific for HC-B27 could therefore be detected by using the dimer or complex. The substance could be detected *in vivo* or *ex-vivo*. In the case of *ex-vivo* detection a sample could be taken from the body of the human or animal, for example a sample of blood or synovial fluid.

The dimer or complex may be used to detect the receptor on the surface of cells or in solution. In the case of measuring the receptor on the surface of cells the

binding may be measured by allowing the dimer or complex to bind to cells from the human or animal, optionally separating cells on the basis of whether or not they have bound the dimer or complex, and measuring the number of cells which have bound the dimer or complex.

If the dimer or complex are fluorescently labelled then the cells which have bound the dimer or complex can be separated and counted using flow cytometry.

The invention therefore provides a dimer or complex which allow a receptor specific for HC-B27 to be detected. The inventors have found that such receptor bearing cells are present at increased levels in patients with AS compared to HLA-B27 positive healthy controls. Therefore the dimer or complex can be used in a diagnostic assay which measures the presence of such a receptor.

Thus the invention provides a method of determining the onset of or predisposition to a spondyloarthropathy comprising measuring the level or detecting the presence of a receptor in the human or animal body which binds to a dimer or complex of the invention. The onset of a spondyloarthropathy includes the onset of a spondyloarthritic disease or a spondyloarthritic attack.

This method can be used to diagnose a spondyloarthropathy in a patient. Alternatively the method may be used to measure the level of the receptor in people who do not have any symptoms. Such people may be predisposed to a spondyloarthropathy or may be suspected of being in the early stages of disease.

The spondyloarthropathy is generally one associated with HLA-B27, and may include ankylosing spondylitis, reactive arthritis, psoriatic arthritis, Reiter's syndrome and juvenile spondyloarthropathy.

The invention further provides a monoclonal antibody which binds the dimer but which does not bind to native HLA-B27. The invention provides use of a dimer of the invention to obtain such an antibody.

The term antibody includes antibody fragments and single chain Fvs which have the required binding properties. The monoclonal antibody can be produced using standard methods for making monoclonal antibodies. Thus the invention provides a method of producing an antibody which comprises:

(i) immunising a human or animal with a dimer or tetramer of the invention.

(ii) extracting from the animal a B cell which produces the antibody.

(iii) obtaining the antibody from the B cell or a cell derived from the B cell, such as a hybridoma.

Such an antibody could be used as a prophylactic or therapeutic agent as administration of the antibody may be expected to interfere with processes dependent on specific binding to HC-B27. Such processes would include the induction of the response which leads to spondyloarthropathy and the action of the HC-B27 specific receptor discussed above.

The antibody can be used in an assay to identify agents which block the binding of the dimer or complex to the antibody. Therefore the invention provides a method of determining in a sample the presence of a substance which inhibits the binding of a dimer or complex of the invention with the antibody comprising:

(i) contacting said sample with said dimer or complex in the presence of said antibody

(ii) determining whether binding of said antibody to said dimer or complex is inhibited.

Thus such an assay can be used to identify a substance which binds specifically to the dimer or complex. Such a substance may also be used as a prophylactic or therapeutic agent in a similar manner to the antibody of the invention.

As discussed above the dimer and complex allow an HC-B27 specific receptor to be detected. Performing the detection in the presence of a candidate substance allows selection of a the substance which inhibits the binding of the dimer or complex to the receptor.

A selected substance which binds specifically to the receptor may be used as a prophylactic or therapeutic agent in a similar manner to the antibody of the invention. Therefore the invention provides a dimer, complex, monoclonal antibody or the selected substance for use in a method of treating a spondyloarthropathy or for use as a prophylactic to prevent the onset of a spondyloarthropathy.

Increased levels of HC-B27 in a human or animal may indicate the onset of or predisposition to spondyloarthropathy. Therefore the invention provides a method of determining the onset of or predisposition to a spondylarthropathy which

comprises measuring the level of or detecting the presence of HC-B27 in the human or animal body or in a sample from the human or animal body.

The level of HC-B27 can be measured using the antibody of the invention or selected substances discussed above which can bind HC-B27.

The dimer of the invention or a tolerising fragment thereof, or a cell of the invention may be used as a tolerising agent. Thus the invention also provides a composition for tolerising a human or animal to HC-B27 which comprises a dimer or complex of the invention or a tolerising fragment thereof; or a cell of the invention. Administration of the composition may lead to a decrease in the level of HC-B27 specific receptor in a human or animal with a spondyloarthropathy. Administration of the composition to a HLA-B27 positive individual without a spondyloarthropathy would tolerise the individual to HC-B27 and therefore decrease the likelihood of the individual developing a HLA-B27 associated spondyloarthropathy.

The tolerising fragment may comprise an epitope, such as a T cell epitope. Methods of tolerisation are well known in the art. Thus the composition would be administered in the appropriate form, amount or route to cause tolerisation.

The invention also provides a polynucleotide which encodes a polypeptide of the dimer of the invention wherein said polypeptide comprises an amino acid sequence which can be recognised and biotinylated by an enzyme. The sequence may be a BirA enzyme specific biotinylation sequence, such as GSLHHILDAQKMVWNHR. The polynucleotide may be DNA or RNA, and may be single or double stranded.

The polynucleotide typically comprises sequence which has homology with the coding sequence of a native HLA-B27 heavy chain gene. Such a homologous sequence may be at least 70% homologous to the native coding sequence, preferably at least 95%, 97% or 99% homologous thereto over a region of at least 20, 40, 60, 100, 200, 300 or more contiguous nucleotides.

A transgenic animal can be produced which expresses a dimer of the invention. The invention thus provides a transgenic animal which has been engineered to express a dimer of the invention which is not a homodimer of the native HLA-B27 heavy chain. Such an animal can be produced using standard

techniques. Such an animal may be a mammal, such as a rodent (e.g. mouse or rat). The transgenic animal may be used as a model for a spondyloarthropathy. The transgenic animal may be used to screen candidate therapeutic agents for a spondyloarthropathy.

The invention provides a substantially isolated receptor which is specific for HC-B27 or substantially isolated cells bearing such a receptor. The receptor or cells may be the ones mentioned above in the specification which are found to be increased in ankylosing spondylitis patients.

The substantially isolated receptor may be substantially free of other cellular components. Thus the receptor may be a substantially pure receptor which is present at at least 50%, 60%, 70%, 80%, 90%, 95% or higher purity by dry weight.

The substantially isolated cells bearing the receptor may be substantially free of cells which do not bear the receptor. Thus the cells may be present at at least 50%, 60%, 70%, 80%, 90%, 95% or higher purity by dry weight.

As discussed above the invention provides therapeutic (including prophylactic), tolerising or diagnostic agents, which are typically the dimer (including the dimer of HLA-B27 heavy chain or the dimeric analogue, e.g. in the form of the multimeric analogue), the substance determined in the method of the invention, the antibody of the invention or a cell of the invention; or an agent which is a therapeutic or tolerising fragment of any of these. In one embodiment a precursor may be administered which provides the agent *in vivo*. Such a precursor is included in the term 'agent'.

The agents are formulated for clinical administration by mixing them with a pharmaceutically acceptable carrier or diluent. For example they can be formulated for topical, parenteral, intravenous, intramuscular, subcutaneous, intraocular or transdermal administration. The agents may be mixed with any vehicle which is pharmaceutically acceptable and appropriate for the desired route of administration. The pharmaceutically carrier or diluent for injection may be, for example, a sterile or isotonic solution such as Water for Injection or physiological saline.

The dose of the agents may be adjusted according to various parameters, especially according to the agent used; the age, weight and condition of the patient to

be treated; the mode of administration used; the conditions to be treated; and the required clinical regimen. As a guide, the amount of agent administered by injection is suitably from 0.01 mg/kg to 30 mg/kg, preferably from 0.1 mg/kg to 10 mg/kg.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The agents of the invention may thus be used in a method of treatment of the human or animal body, or in a diagnostic method practised on the human or animal body. In particular the agents may be used in a method of treating or preventing a spondyloarthropathy, or in a method of determining the onset of, or predisposition to, a spondylarthropathy. The invention also provide the agents for use in a method of manufacture of a medicament for treating or preventing a spondylarthropathy, or for determining the onset of, or predisposition to, a spondylarthropathy. Thus the invention provides a method of preventing or treating a spondylarthropathy, or determining the onset of, or predisposition to, a spondylarthropathy comprising administering to a human or animal an agent of the invention (typically a non-toxic effective amount thereof).

Homologous proteins and nucleic acids are mentioned herein. Methods of measuring nucleic acid and protein homology are well known in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (Devereux *et al* (1984) *Nucleic Acids Research* 12, p.387-395).

Similarly, the PILEUP and BLAST algorithms can be used to line up sequences (for example as described in Altschul S. F. (1993) *J. Mol. Evol.* 36:290-300; Altschul, S. F. *et al* (1990) *J. Mol. Biol.* 215:403-10).

Many different settings are possible for such programs, such as the default settings.

In more detail, the BLAST algorithm is suitable for determining sequence similarity and is described in Altschul *et al* (1990) *J. Mol. Biol.* 215: 403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying

short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a fused gene or cDNA if the smallest sum probability in comparison of the test nucleic acid to a fused nucleic acid is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The invention is further illustrated by the accompanying drawing in which:

Figure 1 shows the identification of HC-B27 dimers by gel filtration. Concentrated refolds of HLA-B27 heavy chain were analysed by gel filtration using a Sephadex75 column on a Pharmacia FPLC system. Running buffer was 20mM Tris (pH8)/150mM NaCl.

Peak (1) represents misfolded aggregate (at the limit of resolution for the column). Peak (2) represents HC-B27 homodimers.

Figure 2 shows a Western blot of HC-B27. HC-B27 complexes folded in the presence (lanes 2 & 4) or absence (lanes 1 & 3) of peptide were analysed by SDS-PAGE and western-blotted with the monoclonal antibody HC10 (specific for HLA B and HLA C heavy chains). SDS-PAGE under reducing conditions identified a band corresponding in size to the extracellular portion of HLA B27 (roughly 31kD) (lane 2). Under non-reducing conditions in the heavy-chain sized band showed an altered mobility (lane 4). For complexes folded in the absence of peptide, degradation products were observed (lanes 1 and 3), indicating that HC-B27 complexes lacking peptide are less stable and are particularly susceptible to degradation.

Figure 3 shows the identification of HLA-B27/β_2m heterodimers by gel filtration. Concentrated refolds of HLA B27 heavy chain and β_2-microglobulin were analysed by gel filtration using a Sephadex75 column on a Pharmacia FPLC system. Running buffer was 20mM Tris (pH8)/150mM NaC1.
Peak (1) represents misfolded aggregate (at the limit of resolution for the column).
Peak (2) represents HLA B27/β_2m heterodimers.

Figure 4 shows peptide elution from HC-B27 complexes. HC-B27 complexes refolded in the presence of the HIV gag epitope were acid-treated and filtered through a 3kD exclusion membrane. Eluates were analysed by HPLC.

Figure 5 shows conformational ELISA of HC-B27 complexes.
Protein complexes were coated onto 96-well plates and assayed for their ability to bind the conformation-specific antibody W6/32 and an irrelevant isotype matched antibody. Indirect ELISA demonstrated comparable W6/32 reactivity for HC-B27 complexes and conventional HLA B27/β_2m heterodimers.

Figure 6 shows that HC-B27 tetramers specifically stain T lymphocytes. Fluorescent HC-B27 tetramers were generated in order to investigate the possibility that a ligand for HC-B27 complexes might be expressed on lymphocytes. Peripheral blood lymphocytes from an HLA B27-positive spondyloarthropathy patient were stained

with PE-conjugated HC-B27 tetramers and FITC-conjugated monoclonal antibodies to the T cell determinant CD3 or the NK cell determinant CD16. Lymphocyte staining was analysed by flow cytometry, the X axis represents HC-B27 tetramer staining and the Y axis antibody staining. Cells positive for tetramer double stained with anti CD3 (a) but not with anti-CD16 (b), thus demonstrating that T cells and not NK cells specifically bind HC-B27.

Figure 7 shows that OKT3 down-regulates HC-B27 staining.

In order to investigate the possibility that HC-B27 might bind the T cell antigen receptor (TCR), peripheral blood lymphocytes from an HLA-B27 positive spondyloarthropathy patient were treated overnight with the monoclonal antibody OKT3. Such treatment is known to result in TCR down-regulation. Treated and untreated cells were stained with HC-B27 and analysed by flow cytometry. The Y axis represents HC-B27 staining. Untreated cells showed higher levels of HC-B27 staining (a) than cells treated with OKT3 (b). These results provide evidence that HC-B27 complexes may be recognised by T cell receptors.

Figure 8 shows that HC-B27 complexes do not present peptide for CTL recognition.

In order to investigate whether HC-B27 complexes could act as an antigen presenting molecule, a CTL line specific for the HIV gag epitope was stained with various HLA-B27 complexes refolded around this peptide. The Y axis shows the percentage of cells staining with the tetramer. Positive staining was observed for standard HLA B27/β_2m heterodimers refolded around the gag epitope. HC-B27 complexes refolded around the gag epitope however, did not stain this line. These results indicate that HC-B27 does not present peptide for CTL recognition but instead is seen as a distinct structural entity.

Figure 9 shows that HC-B27 staining correlates with $\alpha\beta$ but not $\gamma\delta$ TCR expression.

Flow cytometry analysis was performed in order to investigate the possibility that HC-B27 complexes might be recognised by T cells bearing the $\gamma\delta$ TCR. Peripheral blood lymphocytes from a HLA B27-positive spondyloarthropathy patient were

stained with PE-conjugated HC-B27 tetramers and αβ TCR or the γδ TCR-specific, FITC-conjugated monoclonal antibodies. The X axis represents HC-B27 tetramer staining and the Y axis antibody staining. Cells positive for tetramer double stained with anti-αβ TCR antibodies (a) but not with anti-γδ TCR antibodies (b), thus demonstrating that T cells which specifically bind HC-B27 bear the αβ TCR.

Figures 10A and 10B show peripheral blood lymphocytes from (A) an ankylosing spondylitis patient and (B) an HLA B27 positive healthy control stained with fluorogenic HC-B27 complex (X-axis) and antibodies to CD3 (Y axis).

Figures 10C and 10D show (C) peripheral blood and (D) synovial lymphocytes from an ankylosing spondylitis patient stained with fluorogenic HC-B27 complexes (X-axis) and antibodies to CD3 (Y axis).

Figure 11 shows peripheral blood lymphocytes from an ankylosing spondylitis patient stained with fluorogenic HC-B27 complexes (X-axis) and antibodies to either (A) CD4 or (B) CD8 (Y axis).

Figure 12A shows lysates of surface biotinylated cells precipitated using W6/32. Both 45kD heavy chain and 12kD β_2m bands were clearly visible for C1R-HLA B27 cells (Lane 1). A 45kD band was seen in W6/32 precipitates from T2-HLA B27 transfectants (Lane 2), but no corresponding β_2m band was observed. Both bands were absent from untransfected T2 cell precipitates (Lane 3).

Figure 12B shows W6/32 precipitates of T2-HLA B27 lysates analysed by western blot with HC10 antibody. The single heavy chain band seen under reducing conditions (Lane 1), showed an altered mobility under non-reducing conditions (Lane 2) where a very faint dimer and predominant tetramer-sized band were observed.

The following Examples also illustrate the invention:

Example 1

Expression and purification of HLA-B27 heavy chain homodimer

The extracellular domain of the HLA B27-heavy chain (residues 1-275) was expressed in *E. coli* BL21(DE3) pLysS cells (Novagen) from plasmid pLM1-HLA-B27 (a gift from Professor D. Wiley, Harvard University), using the T1 promotor

expressed in *E. coli* BL21(DE3) pLysS cells (Novagen) from plasmid pLM1-HLA-B27 (a gift from Professor D. Wiley, Harvard University), using the T1 promotor expression system. For expression of HLA-B27 heavy chains the bacterial cultures were grown in standard LB broth supplemented with 50mg/ml ampicillin and 0.4% glucose. For large scale expression of the heavy chain a single colony from an LB/amp plate was grown in a 200ml overnight culture of LB/amp. 30ml of this culture was then used to innoculate 1 litre of LB/amp, which was then kept at 37°C with shaking until the O.D. λ_{600} reached 0.7-1.0. Protein expression was induced by addition of IPTG (Melford) to 0.5mM and cells grown for a further 4 hours. Cells were harvested by centrifugation at 3000rpm (JS4.2 rotor, k factor=22548), at 4°C for 30 minutes in a Beckman Centrifuge. Cell pellets were resuspended in 20ml of ice-cold PBS and lysed in 30 second bursts of sonication for a total of 5 mintues. Sonication was performed on ice using a Sonix apparatus. Inclusion bodies were pelleted by centrifugation at 4000rpm (JS4.2 rotor, k factor=12683) for 30 minutes in a Beckman centrifuge and resuspended in Triton Solution (0.5% TritonX100, 50mM Tris pH 8.0, 100mM NaCl, 0.1% NaN_3). Inclusion bodies were transferred to a 20ml polycarbonate tube and pelleted by centrifugation at 15000rpm (JA20 rotor, k factor=1369) for 10 minutes in a Beckman centrifuge. Inclusion body pellets were washed 3 times in Triton solution and finally in Triton-free wash (50mM Tris pH 8.0, 100mM NaCl) to remove detergent. Proteins were then solubilised in 30ml of urea solution (8M Urea, 25mM methylethyl sulphonic acid (MES), 10mM EDTA, 0.1mM DTT) by rotation at 4°C for 48 hours and stored as 1ml aliquots at -70°C.

Example 2

HC-B27 refolding and purification

HC-B27 complexes were refolded by dilution of the urea-denatured heavy chain prepared above into refolding buffer with added peptide (50mM Tris, 200mM L-Arginine, 1mM EDTA, 5mM Reduced Glutathione, 0.5mM Oxidised Glutathione, 30mM peptide). Two naturally presented HLA-B27 restricted epitopes were added as peptide to the refolds - the influenza nucleoprotein (NP) epitope SRYWAIRTR

(residues 383-391) and the HIV gag epitope KRWIIMGLNK (residues 263-272). Refolds were concentrated in an Amicon stir cell under N_2 for FPLC separation. HC-B27 heavy chain homodimers (size 60kD) were purified by gel-filtration on a Pharmacia FPLC system using Sephadex75 or Sephadex200 columns. FPLC running buffer was 20mM Tris (pH8)/150mM NaCl. HC-B27 complexes were concentrated in centriprep filter units (Amicon) and stored at 4°C in 20mM Tris (pH8)/ 150mM NaCl with protease inhibitors at the manufacturer's recommended concentrations (Leupeptin, Pepstatin, PMSF - all supplied by Sigma).

Circular dichroism identified secondary structure within the HC-B27 fraction, confirming that the complexes were not randomly segregated.

Example 3

HC-B27 characterisation

The concentrated refolds were separated by gel filtration using a Sephadex75 or Sephadex200 column on a Pharmacia FPLC system. A dimer-sized peak (corresponding to HC-B27) was observed (Figure 1). The fractions corresponding to this peak were collected and concentrated. HC-B27 was analysed by standard SDS-PAGE electrophoresis through a 15% acrylamide gel. SDS-PAGE gels were western blotted onto Hybond-C membrane, blocked and stained with the HC10 monoclonal antibody (specific for HLA-B and HLA-C heavy chains). The SDS-PAGE and Western blotting were performed according to standard protocols (Molecular Cloning, a laboratory manual. Sambrook *et al*, 1989). HC10 antibody was a gift from H. Ploegh, Harvard University. Western blotting confirmed that the HC-B27 peak corresponded to HLA-B27 heavy chain and not a contaminating *E. Coli* protein (Figure 2). SDS-PAGE analysis of the HC-B27 under reducing conditions identified a band corresponding in size to the extracellular portion of HLA- B27 (roughly 31kD)(Figure 2 lane 2). Under non-reducing conditions the heavy-chain sized band showed an altered mobility with a MW corresponding to roughly 60kD (Figure 2 lane 4). SDS-PAGE and Western blot analysis of HC-B27 refolded in the absence of peptide identified degradation products of heavy chain

(Figure 2 lanes 1 and 3), indicating that HC-B27 complexes lacking peptide are less stable and are particularly susceptible to degradation. Subsequent analysis showed that HC-B27 does not bind the influenza peptide SRYWAIRTR or the HLA-A2 restricted CMV peptide NLVPMVATV.

Example 4

Mutation of residue Cys67 to prevent HC-B27 homodimer formation

A plasmid encoding HLA $B27^{Ser67}$ was generated by PCR mutagenesis of our biotinylation plasmid using a Stratagene Quikchange kit. Residue Cys67 was mutated to serine with the primers shown below using a QuikchangeTM kit (Stratagene) according to the manufacturers instructions.

(i) Forward Primer

5' GAGACACAGATCAGCAAGGCCAAGGC 3'

(ii) Reverse Primer

5' GCCTTGGCCTTGCTGATCTGTGTCTC 3'

Mutation of residue Cys67 to Ser prevented the formation of HC-B27 dimers yet allowed the formation of classical HLA B27 heavy chain/β_2m heterodimers as analysed by gel filtration using a sephadex75 column on a Pharmacia FPLC system (running buffer was 20mM Tris pH 8/150mM Nacl)(Figure 3). The altered mobility of HC-B27 dimers under non-reducing conditions identifies these structures as interchain disulphide-bonded complexes, whilst the Cys67 dependency of their formation would indicate that dimerisation occurs through disulphide bonding of this particular residue.

Example 5

Peptide elution from HC-B27

Following the observation that HC-B27 complexes refolded in the presence of the HIV gag epitope were more stable than those refolded in its absence, peptide

elution studies were performed as described in Elliott *et al*, 1995, J. Exp.Med., vol 181, pp. 1481-91.

Peptides were denatured in 10% acetic acid for 10 minutes at room temperature then centrifuged through a Centricon 3 filter (Amicon). Eluates were analysed by reverse-phase chromatography using a C18 column on a Gilson HPLC system and compared to peptide standards.

Figure 4 shows (a) A single peak (1) was observed for a control sample of peptide in dH20 (b) Two additional peaks (2) and (3) were observed when the control peptide was dissolved in Tris/NaC1 with 10% acetic acid. Peak (3) was common to all samples loaded in Tris/NaC1. An additional peak (2), may result from modification of the peptide by 10% acetic acid. Both peptide peaks (1) and (2) were identified in the eluate of acid-treated HC-B27 (c) but not in that of untreated complexes (d). These results confirm that stably bound peptide can be acid-eluted from HC-B27 and that it is not present as free peptide in untreated samples.

Example 6

ELISA with conformational antibody

The ability of HC-B27 to stably bind peptide would indicate that it can maintain the general conformation of its peptide binding groove. Indirect ELISA analysis of HC-B27 using the monoclonal antibody W6/32 was performed (W6/32 recognises a conformational epitope in the α2 helix of the native HLA peptide binding groove).

A standard indirect ELISA procedure was used (Current Protocols in Immunology, Wiley & Sons, 1995). Proteins were coated onto Maxisorp 96-well plates (Nunc), washed and incubated with first layer antibody at a concentration of 0.5mg/ml in PBS/1%BSA. HRP-conjugated goat anti-mouse IgG antibodies (Sigma) were used as a second layer reagent to bind W6/32. 3,3',5,5'-Tetramethyl-benzidine (TMB) liquid substrate system (Sigma) was used as a developing reagent. O.D. λ=450 was measured on a microtiter counter.

These ELISA studies demonstrated that HC-B27 complexes were W6/32

reactive and therefore that they maintained the general structure of their peptide binding groove. The reactivity of W6/32 to HC-B27 was comparable to the reactivity towards HLA-B27/B_2m heterodimers.

Example 7

Construction of plasmid which expresses HC-B27 fused to a biotinylation sequence

A plasmid encoding the extracellular domains of HLA B27 fused to a specific biotinylation sequence was generated by PCR using an HLA B27-specific N terminal forward primer and a reverse primer encoding residues 269-275 of HLA B27, a glycine linker residue, the BirA specific biotinylation sequence and an appropriate restriction site for cloning into an expression vector. Primer sequences are shown below.

(i) Forward Primer

|EcoRI|

5' TTTGTTGAATTCAGGAGGAAT 3'

(ii) Reverse Primer

|BamHI|

3'CGGGGAGTGGGACTCTACCCTCCCTAGGGACGTAGTATAAGACCTACGTGTCTTTTACCACACCTTA

P L T L R W E G S L H H I L D A Q K M V W N

←HLA B27 Residues 269-275→ ← BirA Specific biotinylation sequence

|HindIII|

GTAGCAATTCGAATAGTGTT 5'

H R Stop

→

PCR was performed using the plasmid pLM1-HLA B27 as a template. The PCR mix also contained 1ng of each primer, 200nM NTPs, Promega purified Pfu polymerase and accompanying buffer to the recommended concentration. PCR conditions were (94°C for 1min, 50°C for 1 min, 68°C for 2 mins) for 30 cycles. PCR products were digested with the restriction enzymes EcoRI and HindIII (Promega) using the supplied

buffers, then gel purified through a 1% low melting point agarose (Promega gel). Appropriately sized PCR products were excised from the gel and extracted using the Wizard™ system (Promega) according to the manufacturers instructions. Digested PCR products were ligated into EcoRI/HindIII digested pLM1 plasmid using T4 DNA ligase (Boehringer) with the supplied buffer. Ligations were transformed into *E. coli* XL1 Blue (Stratagene) for DNA preparation and verification of plasmids.

The plasmid was transformed into *E.coli* BL21(DE3)pLysS (Novagen). The HLA-B27 construct was expressed in *E. coli* BL21(DE3)pLysS cells using the T7 promoter expression system using the protocol described in the previous Examples.

The dimer with the biotinylation sequence was used to perform the experiments of Examples 2 to 6. The results obtained were the same as with the dimer without the biotinylation sequence (results not shown).

Example 8

Biotinylation and tetramerisation

HC-B27 complexes were refolded by dilution and concentrated as described above. Concentrated refolds were transferred into BirA Buffer (100mM Tris, 20mM NaCl, 5mM $MgCl_2$) on a Sephadex PD10 column (Pharmacia). Proteins were then biotinylated using commercially available BirA enzyme, with supplied ATP and biotin (commercial enzyme and substrate mixes are supplied by Avidity Ltd.) at room temperature overnight. Biotinylation was also performed using 7.5mg of in-house purified BirA enzyme with 0.5mM biotin and 5mM ATP. Biotinylated HC-B27 complexes were purified by gel filtration as described above and tetramerised by addition of fluorescence-conjugated streptavidin or extravidin in a 4:1 molar ratio of HC-B27:Streptavidin. FITC and PE conjugates of streptavidin and extravidin are commercially available, we used ExtrAvidin-PE and ExtrAvidin-FITC conjugates (both Sigma). The tetramers formed from the biotinylated dimers are stable at 4°C in BirA buffer for at least 2 months.

Example 9

Flow Cytometry

Fluorescence-conjugated HC-B27 tetramers were generated as described above and used as a reagent for flow cytometric analysis. Peripheral blood and synovial lymphocytes were washed in PBS with 0.1% Bovine Serum Albumin and 0.02% sodium azide (PBS/BSA/Azide) and incubated with 1mg of biotinylated HC-B27 tetramer for 1 hour on ice. If necessary cells were stained with other fluorescence-conjugated and unconjugated antibodies according to standard protocols (Current Protocols in Immunology, Wiley & Sons, 1995). Additional antibodies used were anti-CD3-FITC (DAKO), anti-CD16-FITC (DAKO) anti-TCRαβ-FITC (DAKO), anti-TCRγδ-FITC (Immunotech). After staining, cells were washed as before and resuspended in PBS/BSA/Azide for standard flow cytometric analysis. If necessary cells can be stored in PBS with 0.5% Formalin and 2% Fetal Calf Serum. For analysis of flow cytometric data, live cells were gated and analysed using CellQuest software on a Becton Dickinson FACScan cytometer.

FACS analysis of peripheral blood identified a population of cells staining with HC-B27 tetramers. If HC-B27 is a ligand for T cell receptor the most obvious possibility is that presents peptide for T cell recognition. However, the majority of HC-B27 complexes used for flow cytometry were refolded around the HIV gag epitope yet stained T cells in spondyloarthropathy patients who are known to be HIV negative (Figures 10 and 11). Tetramer positive cells stained predominantly with the CD3 T cell marker but not the CD16 NK cell marker (Figure 6). Treatment of T cells with the OKT3 monoclonal antibody is known to downregulate T cell receptor (TCR) expression. Overnight OKT3 treatment of peripheral blood lymphocytes from am HLA-B27 positive spondyloarthropathy patient resulted in a reduction of HC-B27 staining (Figure 7), providing circumstantial evidence that the receptor for HC-B27 might be TCR.

In order to investigate the ability of HC-B27 to present bound peptide for TCR recognition, a peptide-specific CTL line was stained with complexes (Figure 8). The CTL line was made according to the method in Lalvani et al, 1997, J. Immunol. Methods, vol. 210(1), pp. 65-77. Staining with HC-B27 was much lower than that seen for a classical HLA B27 heavy chain/β_2m heterodimer refolded around the same peptide (about 6.5% of cells for HC-B27 compared to about 0.4% of cells for HLA-B27). This would indicate that if HC-B27 does bind TCR, it is recognised as a distinct structural

entity and not an antigen presenting molecule. In combination with the results listed earlier, the role of peptide might be merely to stabilise the complex.

Due to the association of spondyloarthropathy with the class I allele HLA-B27, CD8 positive T cells have been predicted to play a central role in disease. Despite such predications, CD4 T cells appear to predominate within the affected joints and transgenic studies show that they are involved in the disease process. Our studies described above demonstrate that HC-B27 does not stain peptide-specific CTL. We therefore stained peripheral blood lymphocytes from a spondyloarthropathy patient with HC-B27 and antibodies to either CD4 or CD8. Flow cytometry demonstrated that both CD4 and CD8 T cells were stained with HC-B27. These results might help explain the importance of CD4 T cells in spondyloarthropathy.

Dual colour staining for various lymphocytes markers showed that HC-B27 staining correlated with αβ TCR expression but not γδ TCR expression (Figure 9). Comparison of HC-B27 staining for HLA-B27 positive and HLA- B27 negative healthy controls with HLA-B27 positive spondyloarthropathy patients was performed. The numbers of lymphocytes staining with HC-B27 in healthy HLA-B27 positive controls was very low, but was seen to be dramatically increased in spondyloarthropathy patients (see table below), indicating that HC-B27 may be a target for an autoimmune response during disease. Interestingly, a degree of staining was observed in healthy HLA-B27 negative controls. This might be an indication of the mechanism of disease - for example HC-B27 is involved in an alloreactive response in HLA-B27 negative individuals but would normally be absent in HLA-B27 positives. During disease a breakdown of tolerance may occur, resulting in an autoimmune response to HLA-B27. Analysis of peripheral blood was performed for a single patient on samples taken during an arthritic episode, and following recovery from disease. In accordance with the increased stainings seen for HLA-B27 positive patients, much fewer lymphocytes were seen to stain with HC-B27 following recovery.

Percentage of lymphocytes stained by HC-B27 in patients and healthy controls.

HLA B27 Negative Healthy Controls	HLA B27 Positive Healthy Controls	HLA B27 Patients (Blood)	HLA B27 Patients (Synov Fluid)
0.4	0.2	0.6	12
1.5	0.4	0.6	0.3
0.4	2.2	1.1	1.4
1.2	0.9	1.8	0.9
1.9	0.4	15	2.7
0.5	0.1	2.4	2.3
1.5	0.05		6.3
1.3	0.3		
	0.2		
Mean 1.1	0.6	3.6	3.7
S.D. 0.6	0.7	5.6	4.1

Example 10

Expression of HLA-B27 in T2 cells

HLA-B27 transfected cell lines were studied by immunoprecipitation in order to confirm the presence of HLA-B27 like structures. To study surface expression of MHC class I, 2 x 10^6 cells were labelled with sulfo-NHS biotin then lysed in the presence of 5mM iodoacetimide. Lysates were precipitated using the monoclonal antibody W6/32 (Barnstable *et al.*, (1978) Cell 14: 9-20) with protein A- sepharose (Sigma). C1R-HLA B27 cells were used as a control, and lysates from these cells were shown to contain classical HLA B27/βm complexes. Results are shown in Figure 12. The T2 mutant cell line retains most class I alleles within the cell due to a defect in antigen presentation. However, by immunoprecopitation of surface labelled lysates with W6/32 we showed that W6/32 reactive β_2m-free heavy chains were expressed at the surface of these cells.

W6/32 precipitated material from T2-HLA B27 cells was also analysed by SDS-PAGE. Analysis under reducing and non-reducing conditions indicated that the W6/32-reactive β_2m-free heavy chains expressed in T2-HLA B27 were also disulphide bonded

(no B_2m band was seen). Under reducing conditions, a 45kD single heavy chain band was seen. Under non-reducing conditions a faint dimer band and a predominant tetramer band was seen (this can be explained by disulphide bonding through both Cys67 and a second cysteine in the cytoplasmic tail). These findings indicate that structures similar to the HC-B27 complexes that we previously characterised *in vitro* can be found on living cells *in vivo* under certain conditions.